Our Ref.: 2035-38

U.S. PATENT APPLICATION

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Invention: VIRAL AGENT

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SPECIFICATION

VIRAL AGENT

The present invention relates to the isolation and characterisation of the viral agent responsible for post-transfusional non-A non-B hepatitis (PT-NANBH) and in particular to PT-NANBH viral polypeptides, DNA sequences encoding such viral polypeptides, expression vectors containing such DNA sequences, and host cells transformed by such expression vectors. The present invention also relates to the use of a DNA sequence in a nucleic acid hybridisation assay for the diagnosis of PT-NANBH. The present invention further relates to the use of PT-NANBH viral polypeptides or polyclonal or monoclonal antibodies against such polypeptides in an immunoassay for the diagnosis of PT-NANBH or in a vaccine for its prevention.

Non-A non-B hepatitis (NANBH) is by definition a diagnosis of exclusion and has generally been employed to describe cases of viral hepatitis infection in human beings that are not due to hepatitis A or B viruses. In the majority of such cases, the cause of the infection has not been identified although, on clinical and epidemiological grounds, a number of agents have been thought to be responsible as reviewed in Shih _et al_ (_Prog.Liver Dis_., 1986, _8_, 433-452). In the USA alone, up to 10% of blood transfusions can result in NANBH which makes it a significant problem. Even for PT-NANBH there may be at least several viral agents responsible for the infection and over the years many claims have been made for the identification of the agent, none of which has been substantiated.

European Patent Application 88310922.5 purports to describe the isolation and characterisation of the aetiological agent responsible for PT-NANBH which is also referred to in the application as hepatitis C virus (HCV). A cDNA library was prepared from viral nucleic acid obtained from a chimpanzee infected with PT-NANBH and was screened using human antisera. A number of positive clones were isolated and sequenced. The resulting nucleic acid and amino acid sequence data, which are described in the application, represent approximately 70% of

the 10kb viral genome, and are derived entirely from its 3'-end corresponding to the non-structural coding region.

The present inventors have now isolated and characterised PT-NANBH viral polypeptides by the cloning and expression of DNA sequences encoding such viral polypeptides. Surprisingly, the nucleic acid and amino acid sequence data both show considerable differences with the corresponding data reported in European Patent Application 88310922.5. Overall these differences amount to about 20% at the nucleic acid level and about 15% at the amino acid level but some regions of the sequences show even greater differences. The overall level of difference is much larger than would be expected for two isolates of the same virus even allowing for geographical factors, and is believed to be due to one of two possible reasons.

Firstly, the present inventors and those of the aforementioned European Patent Application used different sources for the nucleic acid used in the cDNA cloning. In particular, the European Patent Application describes the use of chimpanzee plasma as the source for the viral nucleic acid starting material, with the virus having been passaged through a chimpanzee on two occasions. PT-NANBH is of course an human disease and passaging the virus through a foreign host, even if it is a close relative to humans, is likely to result in extensive mutation of the viral nucleic acid. Accordingly, the sequence data contained in European Patent Application 88310922.5 may not be truly representative of the actual viral agent responsible for PT-NANBH in humans. In contrast, the present inventors utilised viral nucleic acid from a human plasma source as the starting material for cDNA cloning. The sequence data thus obtained is much more likely to correspond to the native nucleic acid and amino acid sequences of PT-NANBH.

Secondly, it may be that the viral agent exists as more than one subtype and the sequence data described in the European Patent Application and that elucidated by the present inventors correspond to

separate and distinct subtypes of the same viral agent. Alternatively, it may be that the level of difference between the two sets of sequence data is due to a combination of these two factors.

The present invention provides a PT-NANBH viral polypeptide comprising an antigen having an amino acid sequence that is at least 90% homologous with the amino acid sequence set forth in SEQ ID NO: 3,4,5, 18,19,20,21 or 22, or is an antigenic fragment thereof.

SEQ ID NO : 3,4,5,18,19,20,21 or 22 set forth the amino acid sequence as deduced from the nucleic acid sequence. Preferably, the amino acid sequence is at least 95% or even 98% homologous with the amino acid sequence set forth in SEQ ID NO : 3,4,5,18,19,20,21 or 22. Optionally, the antigen may be fused to an heterologous polypeptide.

Two or more antigens may optionally be used together either in combination or fused as a single polypeptide. The use of two or more antigens in this way in a diagnostic assay provides more reliable results in the use of the assay in blood screening for PT-NANBH virus. Preferably, one antigen is obtained from the structural coding region (the 5'-end) and one other antigen is obtained from the non-structural coding region (the 3'-end). It is particularly preferred that the antigens are fused together as a recombinant polypeptide. This latter approach offers a number of advantages in that the individual antigens can be combined in a fixed, pre-determined ratio (usually equimolar) and only a single polypeptide needs to be produced, purified and characterised.

An antigenic fragment of an antigen having an amino acid sequence that is at least 90% homologous with that set forth in SEQ ID NO : 3,4,5, 18,19,20,21 or 22 preferably contains a minimum of five, six, seven, eight, nine or ten, fifteen, twenty, thirty, forty or fifty amino acids. The antigenic sites of such antigens may be identified using standard procedures. These may involve fragmentation of the polypeptide itself using proteolytic enzymes or chemical agents and

then determining the ability of each fragment to bind to antibodies or to provoke an immune response when inoculated into an animal or suitable in vitro model system (Strohmaier et al, J.Gen.Virol., 1982, 59, 205-306). Alternatively, the DNA encoding the polypeptide may be fragmented by restriction enzyme digestion or other well-known techniques and then introduced into an expression system to produce fragments (optionally fused to a polypeptide usually of bacterial origin). The resulting fragments are assessed as described previously (Spence et al, J.Gen.Virol., 1989, 70, 2843-51; Smith et al, Gene, 1984, 29, 263-9). Another approach is to synthesise chemically short peptide fragments (3-20 amino acids long; conventionally 6 amino acids long) which cover the entire sequence of the full-length polypeptide with each peptide overlapping the adjacent peptide. (This overlap can be from 1-10 amino acids but ideally is n-1 amino acids where n is the length of the peptide; Geysen et al, Proc. Natl. Acad. Sci., 1984, 81, 3998-4002). Each peptide is then assessed as described previously except that the peptide is usually first coupled to some carrier molecule to facilitate the induction of an immune response. Finally, there are predictive methods which involve analysis of the sequence for particular features, e.g. hydrophilicity, thought to be associated with immunologically important sites (Hopp and Woods, Proc. Natl. Acad. Sci., 1981, 78, 3824-8; Berzofsky, Science, 1985, 229, 932-40). These predictions may then be tested using the recombinant polypeptide or peptide approaches described previously.

Preferably, the viral polypeptide is provided in a pure form, i.e. greater than 90% or even 95% purity.

The PT-NANBH viral polypeptide of the present invention may be obtained using an amino acid synthesiser, if it is an antigen having no more than about thirty residues, or by recombinant DNA technology.

The present invention also provides a DNA sequence encoding a PT-NANBH viral polypeptide as herein defined.

The DNA sequence of the present invention may be synthetic or cloned. Preferably, the DNA sequence is as set forth in SEQ ID NO : 3,4,5,18, 19,20,21 or 22.

To obtain a PT-NANBH viral polypeptide comprising multiple antigens, it is preferred to fuse the individual coding sequences into a single open reading frame. The fusion should of course be carried out in such a manner that the antigenic activity of each antigen is not significantly compromised by its position relative to another antigen. Particular regard should of course be had for the nature of the sequences at the actual junction between the antigens. The methods by which such single polypeptides can be obtained are well known in the art.

The present invention also provides an expression vector containing a DNA sequence, as herein defined, and being capable in an appropriate host of expressing the DNA sequence to produce a PT-NANBH viral polypeptide.

The expression vector normally contains control elements of DNA that effect expression of the DNA sequence in an appropriate host. These elements may vary according to the host but usually include a promoter, ribosome binding site,translational start and stop sites, and a transcriptional termination site. Examples of such vectors include plasmids and viruses. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome. For use in E.coli, the expression vector may contain the DNA sequence of the present invention optionally as a fusion linked to either the 5'- or 3'-end of the DNA sequence encoding, for example, β-galactosidase or to the 3'-end of the DNA sequence encoding, for example, the trp E gene. For use in the insect baculovirus (AcNPV) system, the DNA sequence is optionally fused to the polyhedrin coding sequence.

The present invention also provides a host cell transformed with an expression vector as herein defined.

Examples of host cells of use with the present invention include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are *E.coli*, *S.cerevisiae*, *P.pastoris*, Chinese hamster ovary and mouse cells, and *Spodoptera frugiperda* and *Tricoplusia ni*. The choice of host cell may depend on a number of factors but, if post-translational modification of the PT-NANBH viral polypeptide is important, then an eukaryotic host would be preferred.

The present invention also provides a process for preparing PT-NANBH viral polypeptide which comprises cloning or synthesising a DNA sequence encoding PT-NANBH viral polypeptide, as herein defined, inserting the DNA sequence into an expression vector such that it is capable in an appropriate host of being expressed, transforming an host cell with the expression vector, culturing the transformed host cell, and isolating the viral polypeptide.

The cloning of the DNA sequence may be carried out using standard procedures known in the art. However, it is particularly advantageous in such procedures to employ the sequence data disclosed herein so as to facilitate the identification and isolation of the desired cloned DNA sequences. Preferably, the RNA is isolated by pelleting the virus from plasma of infected humans identified by implication in the transmission of PT-NANBH. The isolated RNA is reverse transcribed into cDNA using either random or oligo-dT priming. Optionally, the RNA may be subjected to a pre-treatment step to remove any secondary structure which may interfere with cDNA synthesis, for example, by heating or reaction with methyl mercuric hydroxide. The cDNA is usually modified by addition of linkers followed by digestion with a restriction enzyme. It is then inserted into a cloning vector, such as pBR322 or a derivative thereof or the lambda vectors gt10 and gt11 (Huynh *et al*, *DNA Cloning*, 1985, *Vol 1: A Practical Approach*, Oxford,

IRC Press) packaged into virions as appropriate, and the resulting recombinant DNA molecules used to transform E.coli and thus generate the desired library.

The library may be screened using a standard screening strategy. If the library is an expression library, it may be screened using an immunological method with antisera obtained from the same plasma source as the RNA starting material and also with antisera from additional human sources expected to be positive for antibodies against PT-NANBH. Since human antisera usually contains antibodies against E.coli which may give rise to high background during screening, it is preferable first to treat the antisera with untransformed E.coli lysate so as to remove any such antibodies. It is advantageous to employ a negative control using antisera from accredited human donors, i.e. human donors who have been repeatedly tested and found not to have antibodies against viral hepatitis. An alternative screening strategy would be to employ as hybridisation probes one or more labelled oligonucleotides. The use of oligonucleotides in screening a cDNA library is generally simpler and more reliable than screening with antisera. The oligonucleotides are preferably synthesised using the DNA sequence information disclosed herein. One or more additional rounds of screening of one kind or another may be carried out to characterise and identify positive clones.

Having identified a first positive clone, the library may be rescreened for additional positive clones using the first clone as an hybridization probe. Alternatively or additionally, further libraries may be prepared and these may be screened using immunoscreens or hybridisation probes. In this way, further DNA sequences may be obtained.

Alternatively, the DNA sequence encoding PT-NANBH viral polypeptide may be synthesised using standard procedures and this may be preferred

to cloning the DNA in some circumstances (Gait, Oligonucleotide Synthesis: A Practical Approach, 1984, Oxford, IRL Press).

Thus cloned or synthesised, the desired DNA sequence may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequence is under the control of the functional elements of DNA that effect its expression.

Transformation of an host cell may be carried out using standard techniques. Some phenotypic marker is usually employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the PT-NANBH viral polypeptide may also be carried out using standard techniques.

Antibody specific to a PT-NANBH viral polypeptide of the present invention can be raised using the polypeptide. The antibody may be polyclonal or monoclonal. The antibody may be used in quality control testing of batches of PT-NANBH viral polypeptide; purification of a PT-NANBH viral polypeptide or viral lysate; epitope mapping; when labelled, as a conjugate in a competitive type assay, for antibody detection; and in antigen detection assays.

Polyclonal antibody against a PT-NANBH viral polypeptide of the present invention may be obtained by injecting a PT-NANBH viral polypeptide, optionally coupled to a carrier to promote an immune response, into a mammalian host, such as a mouse, rat, sheep or rabbit, and recovering the antibody thus produced. The PT-NANBH viral polypeptide is generally administered in the form of an injectable formulation in which the polypeptide is admixed with a physiologically acceptable diluent. Adjuvants, such as Freund's complete adjuvant

(FCA) or Freund's incomplete adjuvant (FIA), may be included in the formulation. The formulation is normally injected into the host over a suitable period of time, plasma samples being taken at appropriate intervals for assay for anti-PT-NANBH viral antibody. When an appropriate level of activity is obtained, the host is bled. Antibody is then extracted and purified from the blood plasma using standard procedures, for example, by protein A or ion-exchange chromatography.

Monoclonal antibody against a PT-NANBH viral polypeptide of the present invention may be obtained by fusing cells of an immortalising cell line with cells which produce antibody against the viral polypeptide, and culturing the fused immortalised cell line. Typically, a non-human mammalian host, such as a mouse or rat, is inoculated with the viral polypeptide. After sufficient time has elapsed for the host to mount an antibody response, antibody producing cells, such as the splenocytes, are removed. Cells of an immortalising cell line, such as a mouse or rat myeloma cell line, are fused with the antibody producing cells and the resulting fusions screened to identify a cell line, such as a hybridoma, that secretes the desired monoclonal antibody. The fused cell line may be cultured and the monoclonal antibody purified from the culture media in a similar manner to the purification of polyclonal antibody.

Diagnostic assays based upon the present invention may be used to determine the presence or absence of PT-NANBH infection. They may also be used to monitor treatment of such infection, for example in interferon therapy.

In an assay for the diagnosis of viral infection, there are basically three distinct approaches that can be adopted involving the detection of viral nucleic acid, viral antigen or viral antibody. Viral nucleic acid is generally regarded as the best indicator of the presence of the virus itself and would identify materials likely to be infectious. However, the detection of nucleic acid is not usually as straightforward as the detection of antigens or antibodies since the

level of target can be very low. Viral antigen is used as a marker for the presence of virus and as an indicator of infectivity. Depending upon the virus, the amount of antigen present in a sample can be very low and difficult to detect. Antibody detection is relatively straightforward because, in effect, the host immune system is amplifying the response to an infection by producing large amounts of circulating antibody. The nature of the antibody response can often be clinically useful, for example IgM rather than IgG class antibodies are indicative of a recent infection, or the response to a particular viral antigen may be associated with clearance of the virus. Thus the exact approach adopted for the diagnosis of a viral infection depends upon the particular circumstances and the information sought. In the case of PT-NANBH, a diagnostic assay may embody any one of these three approaches.

In an assay for the diagnosis of PT-NANBH involving detection of viral nucleic acid, the method may comprise hybridising viral RNA present in a test sample, or cDNA synthesised from such viral RNA, with a DNA sequence corresponding to the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22 and screening the resulting nucleic acid hybrids to identify any PT-NANBH viral nucleic acid. The application of this method is usually restricted to a test sample of an appropriate tissue, such as a liver biopsy, in which the viral RNA is likely to be present at a high level. The DNA sequence corresponding to the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22 may take the form of an oligonucleotide or a cDNA sequence optionally contained within a plasmid. Screening of the nucleic acid hybrids is preferably carried out by using a labelled DNA sequence. One or more additional rounds of screening of one kind or another may be carried out to characterise further the hybrids and thus identify any PT-NANBH viral nucleic acid. The steps of hybridisation and screening are carried out in accordance with procedures known in the art.

Because of the limited application of this method in assaying for viral nucleic acid, a preferred and more convenient method comprises

synthesising cDNA from viral RNA present in a test sample, amplifying a preselected DNA sequence corresponding to a subsequence of the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22, and identifying the preselected DNA sequence. The test sample may be of any appropriate tissue or physiological fluid and is preferably concentrated for any viral RNA present. Examples of an appropriate tissue include a liver biopsy. Examples of an appropriate physiological fluid include urine, plasma, blood, serum, semen, tears, saliva or cerebrospinal fluid. Preferred examples are serum and plasma.

Synthesis of the cDNA is normally carried out by primed reverse transcription using random, defined or oligo-dT primers. Advantageously, the primer is an oligonucleotide corresponding to the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22 and designed to enrich for cDNA containing the preselected sequence.

Amplification of the preselected DNA sequence is preferably carried out using the polymerase chain reaction (PCR) technique (Saiki et al, Science, 1985, 230, 1350-4). In this technique, a pair of oligonucleotide primers is employed one of which corresponds to a portion of the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22 and the other of which is located to the 3' side of the first and corresponds to a portion of the complementary sequence, the pair defining between them the preselected DNA sequence. The oligonucleotides are usually at least 15, optimally 20 to 26, bases long and, although a few mismatches can be tolerated by varying the reaction conditions, the 3'-end of the oligonucleotides should be perfectly complementary so as to prime effectively. The distance between the 3'-ends of the oligonucleotides may be from about 100 to about 2000 bases. Conveniently, one of the pair of oligonucleotides that is used in this technique is also used to prime cDNA synthesis. The PCR technique itself is carried out on the cDNA in single stranded form using an enzyme, such as Taq polymerase, and an excess of the

oligonucleotide primers over 20-40 cycles in accordance with published protocols (Saiki et al, Science, 1988, 239, 487-491).

As a refinement of the technique, there may be several rounds of amplification, each round being primed by a different pair of oligonucleotides. Thus, after the first round of amplification, an internal pair of oligonucleotides defining a shorter DNA sequence (of, say, from 50 to 500 bases long) may be used for a second round of amplification. In this somewhat more reliable refinement, referred to as 'Nested PCR', it is of course the final amplified DNA sequence that constitutes the preselected sequence. (Kemp et al, Proc. Natl. Acad. Sci., 1989, 86(7), 2423-7 and Mullis et al, Methods in Enzymology, 1987, 155, 335-350).

Identification of the preselected DNA sequence may be carried out by analysis of the PCR products on an agarose gel. The presence of a band at the molecular weight calculated for the preselected sequence is a positive indicator of viral nucleic acid in the test sample. Alternative methods of identification include those based on Southern blotting, dot blotting, oligomer restriction and DNA sequencing.

The present invention also provides a test kit for the detection of PT-NANBH viral nucleic acid, which comprises

i) a pair of oligonucleotide primers one of which corresponds to a portion of the nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22 and the other of which is located to the 3' side of the first and corresponds to a portion of the complementary sequence, the pair defining between them a preselected DNA sequence;

ii) a reverse transcriptase enzyme for the synthesis of cDNA from test sample RNA upstream of the primer corresponding to the complementary nucleotide sequence of SEQ ID NO : 3,4,5,18,19,20,21 or 22;

iii) an enzyme capable of amplifying the preselected DNA sequence; and optionally;

iv) washing solutions and reaction buffers.

Advantageously, the test kit also contains a positive control sample to facilitate in the identification of viral nucleic acid.

The characteristics of the primers and the enzymes are preferably as described above in connection with the PCR technique.

In an assay for the diagnosis of PT-NANBH involving detection of viral antigen or viral antibody, the method may comprise contacting a test sample with a PT-NANBH viral polypeptide of the present invention, or polyclonal or monoclonal antibody against the polypeptide, and determining whether there is any antigen-antibody binding contained within the test sample. For this purpose, a test kit may be provided comprising a PT-NANBH viral polypeptide, as defined herein, or a monoclonal or polyclonal antibody thereto, and means for determining whether there is any binding with antibody or antigen respectively contained in the test sample. The test sample may be taken from any of the appropriate tissues and physiological fluids mentioned above for the detection of viral nucleic acid. If a physiological fluid is obtained, it may optionally be concentrated for any viral antigen or antibody present.

A variety of assay formats may be employed. The PT-NANBH viral polypeptide can be used to capture selectively antibody against PT-NANBH from solution, to label selectively the antibody already captured, or both to capture and label the antibody. In addition, the viral polypeptide may be used in a variety of homogeneous assay formats in which the antibody reactive with the antigen is detected in solution with no separation of phases.

The types of assay in which the PT-NANBH viral polypeptide is used to capture antibody from solution involve immobilization of the polypeptide onto a solid surface. This surface should be capable of being washed in some way. Examples of suitable surfaces include polymers of various types (moulded into microtitre wells; beads; dipsticks of various types; aspiration tips; electrodes; and optical devices), particles (for example latex; stabilized red blood cells; bacterial or fungal cells; spores; gold or other metallic or metal-containing sols; and proteinaceous colloids) with the usual size of the particle being from 0.02 to 5 microns, membranes (for example of nitrocellulose; paper; cellulose acetate; and high porosity/high surface area membranes of an organic or inorganic material).

The attachment of the PT-NANBH viral polypeptide to the surface can be by passive adsorption from a solution of optimum composition which may include surfactants, solvents, salts and/or chaotropes; or by active chemical bonding. Active bonding may be through a variety of reactive or activatible functional groups which may be exposed on the surface (for example condensing agents; active acid esters, halides and anhydrides; amino, hydroxyl, or carboxyl groups; sulphydryl groups; carbonyl groups; diazo groups; or unsaturated groups). Optionally, the active bonding may be through a protein (itself attached to the surface passively or through active bonding), such as albumin or casein, to which the viral polypeptide may be chemically bonded by any of a variety of methods. The use of a protein in this way may confer advantages because of isoelectric point, charge, hydrophilicity or other physico-chemical property. The viral polypeptide may also be attached to the surface (usually but not necessarily a membrane) following electrophoretic separation of a reaction mixture, such as immune precipitation.

After contacting (reacting) the surface bearing the PT-NANBH viral polypeptide with a test sample, allowing time for reaction, and, where necessary, removing the excess of the sample by any of a variety of means, (such as washing, centrifugation, filtration, magnetism or

capilliary action) the captured antibody is detected by any means which will give a detectable signal. For example, this may be achieved by use of a labelled molecule or particle as described above which will react with the captured antibody (for example protein A or protein G and the like; anti-species or anti-immunoglobulin-sub-type; rheumatoid factor; or antibody to the antigen, used in a competitive or blocking fashion), or any molecule containing an epitope contained in the polypeptide.

The detectable signal may be optical or radioactive or physico-chemical and may be provided directly by labelling the molecule or particle with, for example, a dye, radiolabel, electroactive species, magnetically resonant species or fluorophore, or indirectly by labelling the molecule or particle with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively the detectable signal may be obtained using, for example, agglutination, or through a diffraction or birefringent effect if the surface is in the form of particles.

Assays in which a PT-NANBH viral polypeptide itself is used to label an already captured antibody require some form of labelling of the antigen which will allow it to be detected. The labelling may be direct by chemically or passively attaching for example a radio label, magnetic resonant species, particle or enzyme label to the polypeptide; or indirect by attaching any form of label to a molecule which will itself react with the polypeptide. The chemistry of bonding a label to the PT-NANBH viral polypeptide can be directly through a moiety already present in the polypeptide, such as an amino group, or through an intermediate moiety, such as a maleimide group. Capture of the antibody may be on any of the surfaces already mentioned by any reagent including passive or activated adsorption which will result in specific antibody or immune complexes being bound. In particular, capture of the antibody could be by anti-species or anti-immunoglobulin-sub-type, by rheumatoid factor, proteins A, G and

the like, or by any molecule containing an epitope contained in the polypeptide.

The labelled PT-NANBH polypeptide may be used in a competitive binding fashion in which its binding to any specific molecule on any of the surfaces exemplified above is blocked by antigen in the sample. Alternatively, it may be used in a non-competitive fashion in which antigen in the sample is bound specifically or non-specifically to any of the surfaces above and is also bound to a specific bi- or poly-valent molecule (e.g. an antibody) with the remaining valencies being used to capture the labelled polypeptide.

Often in homogeneous assays the PT-NANBH viral polypeptide and an antibody are separately labelled so that, when the antibody reacts with the viral polypeptide in free solution, the two labels interact to allow, for example, non-radiative transfer of energy captured by one label to the other label with appropriate detection of the excited second label or quenched first label (e.g. by fluorimetry, magnetic resonance or enzyme measurement). Addition of either viral polypeptide or antibody in a sample results in restriction of the interaction of the labelled pair and thus in a different level of signal in the detector.

A suitable assay format for detecting PT-NANBH antibody is the direct sandwich enzyme immunoassay (EIA) format. A PT-NANBH viral polypeptide is coated onto microtitre wells. A test sample and a PT-NANBH viral polypeptide to which an enzyme is coupled are added simultaneously. Any PT-NANBH antibody present in the test sample binds both to the viral polypeptide coating the well and to the enzyme-coupled viral polypeptide. Typically, the same viral polypeptide is used on both sides of the sandwich. After washing, bound enzyme is detected using a specific substrate involving a colour change. A test kit for use in such an EIA comprises:

(1) a PT-NANBH viral polypeptide labelled with an enzyme;

(2) a substrate for the enzyme;

(3) means providing a surface on which a PT-NANBH viral polypeptide is immobilised; and

(4) optionally, washing solutions and/or buffers.

The viral polypeptides of the present invention may be incorporated into a vaccine formulation for inducing immunity to PT-NANBH in man. For this purpose the viral polypeptide may be presented in association with a pharmaceutically acceptable carrier.

For use in a vaccine formulation, the viral polypeptide may optionally be presented as part of an hepatitis B core fusion particle, as described in Clarke *et al* (*Nature*, 1987, *330*, 381-384), or a polylysine based polymer, as described in Tam (*PNAS*, 1988, *85*, 5409-5413). Alternatively, the viral polypeptide may optionally be attached to a particulate structure, such as liposomes or ISCOMS.

Pharmaceutically acceptable carriers include liquid media suitable for use as vehicles to introduce the viral polypeptide into a patient. An example of such liquid media is saline solution. The viral polypeptide itself may be dissolved or suspended as a solid in the carrier.

The vaccine formulation may also contain an adjuvant for stimulating the immune response and thereby enhancing the effect of the vaccine. Examples of adjuvants include aluminium hydroxide and aluminium phosphate.

The vaccine formulation may contain a final concentration of viral polypeptide in the range from 0.01 to 5 mg/ml, preferably from 0.03 to 2 mg/ml. The vaccine formulation may be incorporated into a sterile container, which is then sealed and stored at a low temperature, for example 4^{o}C, or may be freeze-dried.

In order to induce immunity in man to PT-NANBH, one or more doses of the vaccine formulation may be administered. Each dose may be 0.1 to 2 ml, preferably 0.2 to 1 ml. A method for inducing immunity to PT-NANBH in man, comprises the administration of an effective amount of a vaccine formulation, as hereinbefore defined.

The present invention also provides the use of a PT-NANBH viral polypeptide in the preparation of a vaccine for use in the induction of immunity to PT-NANBH in man.

Vaccines of the present invention may be administered by any convenient method for the administration of vaccines including oral and parenteral (e.g. intravenous, subcutaneous or intramuscular) injection. The treatment may consist of a single dose of vaccine or a plurality of doses over a period of time.

The following transformed strains of E.coli were deposited with the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, 61, Colindale Avenue, London, NW9 5HT on the indicated dates:

i) *E. coli* TG1 transformed by pDX113 (WD001); Deposit No. NCTC 12369; 7th December 1989

ii) *E.coli* TG1 transformed by pDX128 (WD002); Deposit No. NCTC 12382; 23rd February 1990.

iii) *E.coli* TG1 transformed by p136/155 (WD003); Deposit No. NCTC ; 28th November 1990.

iv) *E.coli* TG1 transformed by p156/92 (WD004); Deposit No. NCTC ; 28th November 1990.

v) *E.coli* TG1 transformed by p129/164 (WD005); Deposit No. NCTC ; 28th November 1990.

MJS/AC/12th December 1990

vi) E.coli TG1 transformed by pDX136 (WD006); Deposit No. NCTC ; 28th November 1990.

In the Figures, Figure 1 shows a representation of the production of pDX122 described in Example 7, Figure 2 shows a representation of the production of two alternative fused sequences described in Example 17, and Figure 3 shows restriction maps of SEQ ID NO : 21 and 22.

In the Sequence Listing, there are listed SEQ ID NO : 1 to 25 to which references are made in the description and claims.

The following Examples serve to illustrate the invention.

EXAMPLE 1. Synthesis of cDNA

Pooled plasma (160 mls) from two individuals (referred to as A and L) known to have transmitted NANBH via transfusions was diluted (1:2.5) with phosphate buffered saline (PBS) and then centrifuged at 190,000g (e.g. 30,000rpm in an MSE 8x50 rotor) for 5hrs at 4°C. The supernatant was retained as a source of specific antibodies for subsequent screening of the cDNA libraries. The pellet was resuspended in 2mls of 20mM tris-hydrochloride, 2mM EDTA 3% SDS, 0.2M NaCl (2xPK) extracted 3 times with an equal volume of phenol, 3 times with chloroform, once with ether, and then precipitated with 2.5 volumes of ethanol at -20°C. The precipitate was resuspended in 10μl of 10mM tris-hydrochloride, 1mM EDTA at pH 8.0 (TE).

The nucleic acid was used as a template in a cDNA synthesis kit (Amersham International plc, Amersham, U.K.) with both oligo-dT and random hexanucleotide priming. The reaction conditions were as recommended by the kit supplier. Specifically, 1ul of the nucleic acid was used for a first strand synthesis reaction which was labelled with [α-^{32}P]dCTP (Amersham; specific activity 3000Ci/mmol) in a final volume of 20ul and incubated at 42°C for 1 hour. The entire first strand reaction was then used for second strand synthesis reaction,

containing E. coli RNaseH (0.8 U) and DNA polymerase I (23 U) in a final volume of 100ul, incubated at 12°C for 60 minutes then 22°C for 60 minutes. The entire reaction was then incubated at 70°C for 10 minutes, placed on ice, 1 U of T4 DNA polymerase was added and then incubated at 37°C for 10 minutes. The reaction was stopped by addition of 5ul of 0.2M EDTA pH8.

Unincorporated nucleotides were removed by passing the reaction over a NICK column (Pharmacia Ltd, Milton Keynes, U.K.) The cDNA was than extracted twice with phenol, three times with chloroform, once with ether and then 20 μg dextran was added before precipitation with 2.5 volumes of 100% ethanol.

EXAMPLE 2. Production of Expression Libraries

The dried cDNA pellet was resuspended in 5ul of sterile TE and then incubated with 500ng of EcoRI linkers (Pharmacia; GGAATTCC phosphorylated) and 0.5 U of T4 DNA ligase (New England BioLabs, Beverley, MA, USA) in final volume of 10μl containing 20mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP for 3 hours at 15°C. The ligase was inactivated by heating to 65°C for 10 minutes and the cDNA was digested with 180U of EcoRI (BCL, Lewes, U.K.) in a final volume of 100μl at 37°C for 1 hour. EDTA was added to a final concentration of 10mM and the entire reaction loaded onto an AcA34 (LKB) column. Fractions (50μl) were collected and counted. The peak of cDNA in the excluded volume (980 cpm) was pooled, extracted twice with phenol, three times with chloroform, once with ether and then ethanol precipitated.

The ds cDNA was resuspended in 5μl TE and ligated onto lambda gt11 EcoRI arms (Gibco, Paisley, Scotland) in a 10μl reaction containing 0.5U T4 DNA ligase, 66 mM tris-hydrochloride, 10mM $MgCl_2$, 15mM DTT pH 7.6 at 15°C overnight. After inactivating the ligase by heating to 65°C for 10 minutes, 5ul of the reaction were added to an Amersham packaging reaction and incubated at 22°C for 2 hours. The packaged

material was titrated on E. coli strain Y1090 (Huynh et al 1985) and contained a total of $2.6x10^4$ recombinants.

Plating cells (Y1090) were prepared by inoculating 10 mls L-broth with a single colony from an agar plate and shaking overnight at 37°C. The next day 0.5mls of the overnight culture were diluted with 10mls of fresh L-broth and 0.1ml 1M $MgSO_4$ and 0.1ml 20%(w/v) maltose were added. The culture was shaken for 2 hours at 37°C, the bacteria harvested by centrifugation at 5,000g for 10 minutes and resuspended in 5 mls 10mM $MgSO_4$ to produce the plating cell stock. A portion (1ul) of the packed material was mixed with 0.2ml of plating cells, incubated at 37°C for 20 minutes before 3 mls of top agar were added and the entire mixture poured onto a 90mm L-agar plate. After overnight incubation at 37°C plaques were counted and the total number of recombinant phage determined. The remaining packaged material (500ul) was stored at 4°C.

Additional libraries were prepared in a substantially similar manner.

EXAMPLE 3. Screening of Expression Libraries

The initial library described in Example 2 was plated out onto E. coli strain Y1090 at a density of about $5x10^3$ pfu per 140mm plate and grown at 37°C for 2 hours until the plaques were visible. Sterile nitrocellulose filters which had been impregnated with IPTG (isopropylthiogalactoside) were left in contact with the plate for 3 hours and then removed. The filters were first blocked by incubation with blocking solution [3%(w/v)BSA/TBS-Tween(10mM Tris-HCl pH8, 150mM NaCl, 0.05%(v/v) Tween 20) containing 0.05% bronidox] (20mls/filter) and then transferred to binding buffer [1%(w/v)BSA/TBS/Tween containing 0.05% bronidox] containing purified (by ion-exchange chromatography) antibodies from pooled A & L plasma (20μg/ml). After incubation at room temperature for 2 hours the filters were washed three times with TBS-Tween and then incubated in binding buffer

containing biotinylated sheep anti-human (1:250). After 1 hour at room temperature the filters were washed 3 times with TBS/Tween and then incubated in binding buffer containing streptavidin/peroxidase complex (1:100). The signal developed with DAB. Positive signals appeared as (coloured) plaques.

Out of a total of 2.6×10^4 plaques screened, 8 positives were obtained on the first round screen. Using the filters as a template, the regions of the original plates corresponding to these positive signals were picked off using a sterile pasteur pipette. The agar plugs were suspended in 0.1 ml of SM buffer and the phage allowed to diffuse out. The titre of phage from each plug was determined on E. coli strain Y1090. The phage stock from each plug was then re-screened as before on individual 90mm plates at a density of about 1×10^3 pfu per plate. Of 8 first round positives, one was clearly positive on the second round, i.e. >1% of plaques positive, this was called JG2. This corresponds to a positive rate of $40/10^6$ in the library.

This and other positive phage identified in an similar way from other cDNA libraries described in Example 2 were then purified by repeated rounds of plaque screening at lower density (1-200 pfu/90mm plate) until 100% of the plaques were positive with the A&L antibody screen. Three such recombinant phage were JG1, JG2 and JG3.

EXAMPLE 4. Secondary Screening of JG1, JG2 and JG3 with Serum Panels

Each of the recombinant phage, JG1, JG2 and JG3, were plaque purified and stored as titred stocks in SM buffer at 4°C. These phage were mixed (1:1) with a stock of phage identified as negative in Example 3 and mixture used to infect E. coli strain Y1090 at 1000 pfu per plate. Plaque lifts were taken and processed as described in Example 3 except that the filters were cut into quadrants and each quadrant was incubated with a different antibody; these were A&L antibodies (20μg/ml); A plasma (1:500); L plasma (1:500) and H IgG (20μg/ml). H

is a patient expected to be positive for PT-NANBH antibodies because he was a haemophiliac who had received non-heat-treated Factor VIII. At the end of the reaction each filter was scored blind as positive (when there were clearly two classes of signal) or negative (when all plaques gave the same signal). This could be a subjective judgement and so the scores were compared and only those filters where there was a majority agreement were taken as positive. The results are presented in Table 1.

TABLE 1

	A&L	A	L	H
JG1	+	+	-	-
JG2	+	+	+	+
JG3	+	+	+	+

JG1 appeared only to react with antibodies from patient A and not L or H; this is not what would be expected of a true PT-NANBH related recombinant polypeptide and so JG1 was dropped from the analysis. However both JG2 and JG3 gave clear positive reactions with three PT-NANBH sera A, L and H; these were analysed further.

The type of analysis described above was repeated for JG2 and JG3 except that the filters were cut into smaller portions and these were incubated with panels of positive and negative sera. The panels of positive sera comprised one panel of 10 haemophiliac sera and one panel of 9 intravenous drug addict (IVDA) sera. These represented the best source of positive sera even though the actual positive rate was unknown. The panel of negative sera was obtained from accredited donors who have been closely monitored over many years by the North London Blood Transfusion Centre, Deansbrook Road, Edgware, Middlesex, U.K. and have never shown any sign of infection with a variety of agents including PT - NANBH. The results are presented in Tables 2 & 3.

TABLE 2

	I.D.	JG2	JG3
IVDAs	V19146	4/4	0/5
	V27083	2/4	0/5
	V29779	0/4	0/5
	V12561	0/5	4/5
	V15444	3/4	5/5
	V18342	4/4	0/5
	V8403	3/4	0/5
	V20001	4/4	0/5
	V21213	3/4	0/5
Haemophiliacs	M1582	4/4	4/5
	M1581	5/5	5/5
	M1575	3/5	0/5
	M1579	5/5	5/5
	M1585	3/5	0/5
	M1576	1/5	1/5
	M1580	1/5	0/5
	M1578	1/5	0/5
	M1587	1/5	3/5
	M1577	2/5	1/5

Positives are underlined.

TABLE 3

	IVDA	Haemophiliac	Accredited Donor
JG2	6/9(66%)	5/10(50%)	0/10(0%)
JG3	2/9(22%)	4/10(40%)	0/10(0%)
JG2+JG3	1/9(11%)	3/10(30%)	0/10(0%)
JG2 or JG3	7/9(77%)	6/10(60%)	0/10(0%)

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These data are consistent with the hypothesis that both recombinants are expressing polypeptides associated with an agent responsible for PT-NANBH and that these polypeptides are not identical but may share some antigenic sites.

EXAMPLE 5. Restriction Mapping and DNA Sequencing of JG2 and JG3

A portion (10μl) of the phage stocks for both JG2 and JG3 was boiled to denature the phage and expose the DNA. This DNA was then used as a template in a PCR amplification using Taq polymerase; each reaction contained the following in a final volume of 50ul:- 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, pH 8.3 at 25°C plus oligonucleotide primers d19 and d20 (SEQ ID NO : 1 and 2 respectively; 200ng each); these primers are located in the lambda sequences flanking the Eco RI cloning site and therefore prime the amplification of anything cloned into this site.

A portion of the reaction was analysed on a 1.0% agarose gel and compared to markers. Amplification of JG2 produced a fragment of approximately 2Kb; JG3 one of approximately 1Kb. The remaining reaction mix was extracted with phenol/chloroform in the presence of 10mM EDTA and 1% SDS and the DNA recovered by ethanol precipitation. The amplified material was then digested with 20U of EcoRI for 60 minutes at 37°C and separated on a 1.0% LGT agarose gel in TAE. The fragments were reduced in size as expected and were eluted and purified using Elutips (S&S). The JG2 and JG3 inserts were ligated with EcoRI digested pUC13 and transformed into E. coli strain TG1. Recombinants were identified as white colonies on X-gal/L-Amp plates (L-Agar plates supplemented with 100 μg/ml ampicillin, 0.5 mg/ml X-gal) and were checked by small-scale plasmid preparations and EcoRI restriction enzyme digestion to determine the size of the insert DNA. The recombinant plasmid containing the JG2 insert was called DM415 and that containing the JG3 insert was called DM416.

The sequence of the JG2 insert was determined by direct double-stranded sequencing of the plasmid DNA and by subcloning into M13 sequencing vectors such as mp18 and mp19 followed by single-stranded sequencing. The sequence of the JG3 insert was similarly determined. The resulting DNA and deduced aminoacid sequences are set forth in SEQ ID NO : 3 and 4.

EXAMPLE 6. Expression of PT-NANBH Polypeptide in E.coli

The plasmid pDM416 (5ug) was digested with EcoRI (20U) in a final volume of 20ul and the 1Kb insert recovered by elution from a 1% LGT agarose gel. This material was then "polished" using Klenow fragment and a dNTP mix to fill in the EcoRI overhanging ends. The DNA was recovered by ethanol precipitation following extraction with phenol/chloroform. The blunt-ended fragment was ligated into SmaI cleaved/phosphatased pDEV107 (a vector which permits cloning at the 3' end of lac Z) and then transformed into E. coli TG1 cells. There was a 30-fold increase in colonies over a vector-alone control. Transformants containing the required recombinant plasmid were identified by hybridisation with a radioactive probe produced by PCR amplification of the JG3 recombinant. Twelve colonies were analysed by restriction enzyme digestion (SalI) of plasmid mini-preparations to determine the orientation of the insert. A quarter of these recombinants were in the correct orientation to express the PT-NANBH sequence as a fusion with β-galactosidase. One of these (pDX113) was taken for further analysis.

A colony of pDX113 was used to inoculate 50 mls L-broth, grown at 37°C with shaking to mid-log phase and expression induced by addition of 20mM IPTG. After 3 hours the cells were harvested by centrifugation at 5,000g for 20 minutes, resuspended in 50 mls PBS and repelleted. The pelleted cells were resuspended in 5 mls of buffer (25mM Tris-HCl, 1mM EDTA, 1mg/ml lysozyme, 0.2%(v/v) Nonidet-P40, pH8.0) per gram of pellet and incubated at 0°C for 2 hours. The released bacterial DNA

was digested by addition of DNase I and $MgSO_4$ to final concentrations of 40ug/ml and 2mM respectively to reduce viscosity.

This crude lysate was analysed by PAGE and the pattern of proteins stained with Coomassie blue. A protein of approximately 150kD was induced in bacteria containing pDX113 and this protein was estimated to account for 10-15% of the total protein. Similar gels were transferred to PVDF membrane (GRI, Dunmow, Essex, U.K.) and the membranes incubated with PT-NANBH-positive and negative sera; the 150kD protein reacted with the A and L sera but not normal human serum. Control tracks containing lysate from _E. coli_ expressing β-galactosidase did not react with A, L or normal human sera.

Urea was added to the crude lysate to a final concentration of 6M and insoluble material removed by centrifugation. The 6M urea extract was used to coat microtitre wells directly for 1 hour at 37°C. The wells were washed three times with double-distilled water and then blocked by addition of 0.25ml of 0.2% BSA per well containing 0.02% NaN_3 for 20 minutes at 37°C. The plate was then aspirated. Control plates coated with a crude lysate of a β-galactosidase-producing _E. coli_ strain (pXY461) were produced in the same way. These plates were used in ELISA assays as described in Example 10.

EXAMPLE 7. Expression of PT-NANBH Polypeptide in Insect Cells

The PT-NANBH insert from JG3, isolated as described in Example 5, was cloned in-frame with the first 34 nucleotides of polyhedrin in the vector pAc360 (Luckow and Summers, _Biotechnology_, 1988, _6_, 47-55), utilising our knowledge of the reading frame of the _lac_Z gene in the gt11 vector. Oligonucleotides were synthesised which were able to hybridise to gt11 sequences flanking the EcoRI cloning site and which would enable the amplification of the insert by PCR. These oligonucleotides included BamHI restriction sites suitably placed to allow direct cloning into the BamHI site of pAc360, placing the

inserted gene in-frame with the amino terminal sequences of polyhedrin.

A small amount of the gtll recombinant JG3 was boiled to expose the DNA and then used in a PCR amplification containing the oligonucleotide primers d75 and d76 (SEQ ID NO : 6 and 7; 200mg) and 0.5U of Taq polymerase.

After amplification, the reaction was extracted with an equal volume of phenol/chloroform, ethanol precipitated and digested with 10U BamHI in a final volume of 30ul. The amplified fragment was resolved on a 1% agarose gel, eluted and ligated into BamHI-digested pAc360 to produce the transfer construct pDX119. The recombinant plasmid (2ug) and wild-type AcNPV DNA (1ug) were co-transfected into insect cells by calcium phosphate precipitation. Inclusion negative recombinant virus was selected by visual screening. After three rounds of plaque purification, the recombinant virus (BHC-5) was expanded and expression of recombinant protein in insect cells was assessed by SDS-PAGE, Western blot and ELISA. An abundantly expressed protein of approximately 70kD in produced in infected cells. This protein is reactive with PT-NANBH sera by Western blot and ELISA.

A further baculovirus recombinant (BHC-7) was constructed to include JG2 sequences additional to the JG3 sequences present in BHC-5, as depicted in Figure 1. The PT-NANBH sequences present in JG2 were amplified and cloned into the pAc360 vector as described above to produce pDX118 and the appropriate Bam HI/Sal I fragments of pDX119 and pDX118 were linked together in that order in pAc360 to produce the transfer construct pDX122.

Recombinant plasmids were identified by hybridisation and orientation of inserted DNA determined by restriction enzyme analysis. Recombinant virus was produced as described above and the expressed protein analysed by SDS-PAGE, Western blot and ELISA. A very abundant

(40% total cell protein) 95kDa polypeptide which reacted with PT-NANBH sera was found in infected cells.

EXAMPLE 8. Purification of DX113 Polypeptide

E. coli strain TG1 containing the plasmid pDX113 (designated strain WDL001) was grown and induced in a 1.5 litre fermenter (model SET002, SGI, Newhaven, East Sussex, U.K.) at 37°C for 5 hours. The cells were harvested by centrifugation at 5,000g for 20 minutes and treated as follows.

a) Extraction.

The wet cells are resuspended (1:20, w/v) in Buffer A (50mM Tris-HCl, 50mM NaCl, 1mM EDTA, 5mM DTT, 10%(v/v) glycerol, pH8.0). Lysozyme was added at 5mg solid per ml of suspension and the mixture left at 4°C. After 15 minutes, the mixture was sonicated (6um peak-to-peak amplitude) on ice for a total of 3 minutes (6x 30 sec bursts). DNase I was added at 4ug per ml suspension and the mixture left for a further 30 minutes. The suspension was centrifuged for 20 minutes at 18,000g(max) and the supernatant discarded.

The pellet was resuspended in buffer B (25mM Hepes, 4M urea, 5mM DTT, pH 8.0) at a ratio of 1:6 (w/v) to obtain a fine suspension. This was centrifuged at 18,000g(max) for 20 minutes and the supernatant discarded. The pellet was resuspended in buffer C (25mM Hepes, 8M urea, 2mM DTT, pH 8.0) at a ratio of 1:6 (w/v); before suspension the following are added:- leupeptin (1ug/ml), pepstatin (1ug/ml) and E64 (1ug/ml). The suspension was centrifuged at 18,000g(max) for 30 minutes and the supernatant decanted and kept. The pellet was resuspended in 25mM Hepes, 1% SDS pH 8.0.

b) Chromatography.

The supernatant from the 8M urea fraction was diluted 1:5 (v/v) in 25mM Hepes, 8M urea, 2mM DTT, pH 8.0 and fractionated on a 7ml Q-Sepharose column. Proteins were eluted via a salt gradient of 0-1M NaCl. The chromatography and data manipulation were controlled by an FPLC (Pharmacia). DX113 elutes at approximately 500mM NaCl and is virtually homogeneous by SDS Page and Western blot analysis.

EXAMPLE 9. Purification of BHC-5 Polypeptide

Sf9 cells ($2x10^9$) were infected with a stock of the BHC-5 recombinant virus (moi 5). After incubation at 28^oC for 2 days the cells were harvested by centrifugation and then processed as follows.

a) Extraction.

The wet cell mass (1.2g) was resuspended in 6mls of buffer A (25mM Hepes, 5mM DTT, leupeptin 1μg/ml, pepstatin 1μg/ml, E64 1μg/ml pH 8.0). The resuspended cells were placed on ice and sonicated for 3 x 15 seconds bursts (6μm peak-to-peak amplitude) interspersed with 30 second rest periods. The sonicated suspension was centrifuged at 18,000g(max) for 20 minutes and the supernatant discarded. The pellet was resuspended in buffer A plus 4M urea (6mls) and centrifuged at 18,000g (max) for 20 minutes. The supernatant was discarded and the pellet re-extracted with buffer A plus 8M urea (6ml). After centrifugation at 18,000g (max) for 30 minutes the supernatant was retained and diluted 1:6 in buffer A plus 8M urea. This extract was chromatographed on a mono-Q column equilibrated in the same buffer. The column was eluted via a salt gradient (0-1.0M NaCl) over 12 column volumes. BHC-5 eluted at approximately 0.45 - 0.55m NaCl and was greater than 90% pure as judged by SDS-PAGE. The yield, was approximately 70%.

EXAMPLE 10.

Performance of DX113 and BHC-5 and 7 Polypeptides in an ELISA

Microelisa plates (96 well, Nunc) were directly coated in 50mm bicarbonate buffer (50mM sodium bicarbonate and 50mM sodium carbonate, titrated to pH 9.5) with either a crude 6M urea lysate of BHC-5 or with purified pDX113. Plates were blocked with 0.2% BSA and then incubated for 30 minutes at 37°C with sera diluted 1:20 (baculo) or 1:100 (*E. coli*). After washing in Tween-saline (0.85% saline, 0.05% Tween 20, 0.01% Bronidox) plates were incubated with peroxidase-conjugated goat anti-human immunoglobulin (1:2000) for 30 minutes at 37°C. Plates were then washed in Tween-saline and colour developed by adding the chromogenic substrate TMB (tetramethyl benzidine-HCl) (100μl/well) and incubating for 20 minutes at room temperature. The reaction was stopped with 50μl 2M sulphuric acid and the OD450 determined (Table 4;)

TABLE 4

Indirect anti-human Ig format ELISA for the detection of NANB antibody

	Baculo BHC-5 (Solid phase)	E.coli DX113 (Solid phase)
	>2	1.670
	1.855	1.531
	1.081	1.015
Sera from high risk	1.842	1.558
patients positive	0.526	0.638
in the assay	>2	1.516
	1.823	1.602
	1.779	1.318
	1.122	0.616
	1.686	1.441

	0.259	0.205
	0.158	0.120
	0.298	0.209
Sera from high risk	0.194	0.111
patients negative	0.282	0.181
in the assay	0.263	0.165
	0.184	0.163
	0.121	0.099
	0.243	0.104
Accredited donor	0.224	0.119

Sera from patients at high risk of PT-NANB infection (IVDA's, haemophiliacs) were assayed as described; all data are expressed as OD450 readings with the accredited donor as a negative control. Of this particular group of sera 10/19 are positive on both solid phases.

Additionally purified DX113 was conjugated to alkaline phosphatase using SATA/maleimide reduction and an immunometric assay was established. Known NANB positive and negative sera were diluted as indicated in accredited donor serum and added to a BHC-7 coated solid phase. Either simultaneously or after incubation (30 minutes at 37°C) the DX113 conjugate was added (50μl, 1:2000). After incubation at 37°C for 30 minutes, plates were washed with 50mM bicarbonate buffer and colour developed using the IQ Bio amplification system and the OD492 determined (Table 5)

TABLE 5

Immunometric (labelled polypeptide) ELISA for the detection of NANB antibody

Positive in Assay	Negative in Assay	Accredited donor
>2	0.217	0.234
0.821	0.252	
>2	0.214	
0.542	0.257	
0.876	0.308	
1.583	0.278	
>2	0.296	
>2	0.273	
1.830	0.262	
>2	0.251	

Thus with either assay format - antiglobulin or immunometric - all the high risk samples gave concordant results.

EXAMPLE 11 - Vaccine Formulation

A vaccine formulation may be prepared by conventional techniques using the following constituents in the indicated amounts:

PT-NANBH Viral polypeptide	> 0.36 mg
Thiomersal	0.04-0.2 mg
Sodium Chloride	< 8.5 mg
Water	to 1ml

EXAMPLE 12 -

Production of Monoclonal Antibodies to PT-NANBH Polypeptides

The DNA insert from DM415 was sub-cloned into the baculovirus transfer vector p36C and recombinant virus produced by a method essentially similar to that described in Example 7. The recombinant virus was called BHC-1 and expressed very low levels of PT-NANBH-specific protein. Sf-9 cells ($5x10^7$ cells/ml) infected with BHC-1 were lysed in PBS containing 1% (v/v) NP40 and spun at 13000g for 2 minutes. The supernatant was passed over Extractigel-D (Pierce Chemicals) to remove detergent and then mixed as a 1:1 emulsion with Freund's complete adjuvant. Mice were injected subcutaneously with 0.1ml of emulsion (equivalent to $5x10^6$ cells). At 14 and 28 days post-injection, the mice were boosted by intraperitoneal injection of 0.1ml (equivalent to $5x10^6$ cells) of a detergent-free extract of BHC-5-infected Sf-9 cells: BHC-5 contains the DNA insert of DM416. Test tail bleeds were taken and assayed for anti-PT-NANBH activity in an ELISA (Example 10). Two mice with a PT-NANBH-specific response were further boosted by i.v. injection with a detergent-free extract of BHC-7-infected Sf-9 cells; BHC-7 contains a DNA insert produced by ligating together the overlapping regions of DM415 and DM416 (Example 7). The spleens were removed three days later.

Spleen cells were fused with NSo myeloma cells in the presence of PEG1500 by standard techniques. The resulting hybridoma cells were selected by growth in HAT (hypoxanthine, aminopterin, thymidine) medium. At 10-14 days post-fusion, supernatants were screened for anti-PT-NANBH activity by ELISA. Wells which showed reactivity with both DX113 and BHC-7 antigens (Example 10) were identified and individual colonies were transferred to separate wells, grown and re-tested. Wells which showed specific reactivity at this stage were further cloned at limiting dilution to ensure monoclonality.

EXAMPLE 13. Detection of PT-NANBH Viral Nucleic Acid in Seropositive Patients

Sera: Donation samples from 1400 donors, enrolled into a prospective study of post-transfusion hepatitis, were frozen at -20°C.

Pre-transfusion and serial post-transfusion samples from the 260 recipients were similarly stored. The post-transfusion samples were collected fortnightly until 3 months, monthly until 6 months and 6 monthly thereafter, until 18 months. Frozen donor and recipient sera from three incidents of PT-NANBH that occurred in 1981 were also available for study. The diagnosis of PT-NANBH was based on a rise in serum alanine amino transferase (ALT) to exceed 2.5 times the upper limit of normal in at least two separate post-transfusion samples. Other hepatotropic viruses were excluded by serological testing and non-viral causes of hepatocellular injury were excluded by conventional clinical and laboratory studies.

Immunoassay: Serum samples were tested retrospectively for the presence of antibodies to HCV (C100 antigen) with the Ortho Diagnostics ELISA kit used in accordance with the manufacturer's instructions. Repeatedly reactive sera were titrated to end points in a human serum negative for anti-C100.

Detection of PT-NANBH Viral Sequences: Serum or plasma RNA was extracted, reverse transcribed, and amplified as described below. The reverse transcription/PCR oligonucleotide primers were derived from the nucleotide sequence of the JG2 clone isolated in EXAMPLE 3, and synthesised on an Applied Biosystems 381A synthesiser. The sequences of the four oligonucleotide primers were as follows:

Designation	SEQ ID NO :	Product Size
d94 sense	8	729bp
d95 antisense	9	
N1 sense	10	402bp
N2 antisense	11	

(i) RNA Extraction

5-50μl of serum (or plasma) was made up to 200μl by adding sterile distilled water. The 200μl sample was added to an equal volume of 2 x PK buffer (2 x PK = 0. 2M TrisCl, pH7.5, 25mM EDTA, 0.3M NaCl, 2% w/v SDS, proteinase K 200μg/ml), mixed and incubated at 37°C for 40 minutes. Proteins were removed by extracting twice with phenol/chloroform and once with chloroform alone. 20μg glycogen were added to the aqueous phase and the RNA then precipitated by addition of 3 volumes of ice-cold absolute ethanol. After storage at -70°C for 1 hour the RNA was pelleted in an Eppendorf centrifuge (15 minutes, 14000 rpm, 4°C). The pellet was washed once in 95% ethanol, vacuum desiccated and dissolved in 10μl of sterile distilled water. RNA solutions were stored at -70°C.

(ii) cDNA Synthesis

A 10μl mixture was prepared containing 2μl of the RNA solution, 50ng of the synthetic oligonucleotide d95, 10mM Hepes-HCl pH6.9 and 0.2mM EDTA pH8.0. This 10μl mix was overlayed with 2 drops of mineral oil, heated for 2 minutes in a water bath at 90°C and cooled rapidly on ice. cDNA synthesis was performed after adjusting the reaction to contain 50mM Tris-HCl pH7.5, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 0.5mM each of dATP, dCTP, dGTP and dTTP, 20 units of RNase inhibitor (Pharmacia) and 15 units of cloned MLV reverse transcriptase (Pharmacia) in a final volume of 20μl. The 20μl mix was incubated at 37°C for 90 minutes. Following synthesis the cDNA was stored at -20°C.

(iii) "Nested" PCR

Throughout this study false positive PCR results were avoided by strict application of the contamination avoidance measures of Kwok and Higuchi (Nature, 1989, 339, 237-238).

a) Round 1

The polymerase chain reaction was performed in a 50μl mix containing 10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% w/v gelatin, 1 Unit Recombinant Taq DNA polymerase (Perkin Elmer Cetus), 200μM each dNTP, 30ng of each 'outer' primer (d94 and d95; SEQ ID NO : 8 and 9 respectively) and 5μl of the cDNA solution. After an initial 5 minute denaturation at 94^{o}C, 35 cycles of 95^{o}C for 1.2 minutes, 56^{o}C for 1 minute, 72^{o}C for 1 minute were carried out, followed by a final 7 minute extension at 72^{o}C (Techne PHC-1 Automated Thermal Cycler).

b) Round 2

The reaction mix was as described above for Round 1 but 125ng of each 'inner' primer, N1 and N2 (SEQ ID NO : 10 and 11 respectively), was used instead of the 'outer' primers d94 and d95. A 1μl aliquot of the Round 1 PCR products was transferred to the Round 2 50μl reaction mix. 25 cycles of 95^{o}C for 1.2 minutes, 46^{o}C for 1 minute, 72^{o}C for 1 minute were performed followed by a 7 minute extension at 72^{o}C.

c) Analysis

20μl of the Round 1 and Round 2 PCR products were analysed by electrophoresis on a 2% agarose gel. Bands were visualised by ethidium bromide staining and photographed at 302nm.

Predictive Value of Anti-HCV Serology and PCR in the Prospective Study: Six of the 1400 donors (0.43%) enrolled into the prospective study were found to have antibodies to C100 in their serum. Of these six antibody positive donors only one (donor D6) proved to be infectious as judged by the development of PT-NANBH and C100 seroconversion in a recipient (recipient R6) - see Table 6 below.

Viral sequences were detected by PCR in the serum of donor D6 but not in any of the other five seropositive donor sera. The recipient R6 who developed PT-NANBH had also received blood from seven other donors (D7 to D13). Sera from these donors were tested and found to be both antibody negative and PCR negative.

TABLE 6

DONOR/RECIPIENT DATA SUMMARY : PROSPECTIVE STUDY

DONORS			RECIPIENTS		
Donor	anti-HCV	PCR	Recipient	PT-NANBH	Anti-HCV seroconversion
D1	+	-	R1	No	No
D2	+	-	R2	No	No
D3	+	-	R3	No	No
D4	+	-	R4	No	No
D5	+	-	R5	No	No
D6	+	+			
D7	-	-			
D8	-	-			
D9	-	-	R6	Yes*	Yes+
D10	-	-			
D11	-	-			
D12	-	-			
D13	-	-			

* incubation period 1 month

\+ Seroconversion occurred at 5 months post-transfusion

Example 14 -

Isolation and Expression of Additional PT-NANBH DNA Sequences

The lambda gt11 libraries prepared in Example 2 were also screened with sera from patients with a high risk for PT-NANBH but which did not react with the viral antigens, DX113, BHC-5 and BHC-7, the reasoning being that they might well contain antibodies which recognise different antigens. The sera, PJ-5 (The Newcastle Royal Infirmary, Newcastle), Birm-64 (Queen Elizabeth Medical Centre, Birmingham), PG and Le (University College and Middlesex School of Medicine, London) met this criterion and were used to screen the libraries following the same procedure as described in Examples 3 and 4. A number of recombinants were thus identified, none of which cross-hybridised with probes made from JG2 and JG3. One of the recombinants, BR11, identified by reaction with PJ-5, was selected for further analysis.

The clone, BR11, contained an insert of approximately 900bp which was amplified by PCR using the d75 and d76 primers [SEQ ID NO : 6 and 7) as described in Example 7. The amplified sequence was directly cloned into the baculovirus vector pAc360 to form pDX128 containing an open reading frame in phase with the first 11 amino acids of polyhedrin. Recombinant baculovirus stocks (designated BHC-9) were produced following the procedure described in Example 7. Insect cells were infected with purified recombinant virus and a polypeptide of approximately 22kD was obtained in radiolabelled cell extracts.

The amplified insert of BR11 was also cloned into pUC13 and M13 phage vector for sequencing; the DNA and aminoacid sequence data are presented in SEQ ID NO : 5. The insert contains 834bp plus the EcoRI linkers added during cloning.

Example 15 - Performance of BHC-9 Polypeptide in an ELISA

An ELISA was established using microtitre wells coated with BHC-9-infect cell extract and an anti-human Ig conjugate detection system following the procedure as described in Example 10. A panel of high-risk sera were assayed in parallel against BHC-7 and BHC-9 and were also examined by PCR using the procedure described in Example 13. The results are shown in Table 7 in which positive samples are underlined.

TABLE 6

Number	PCR	BHC-7	BHC-9
1	+	2.09	2.00
2	+	2.09	2.00
3	+	1.89	1.37
4	+	1.57	0.27
5	+	1.26	2.00
6	+	0.91	2.00
7	-	0.90	0.51
8	+	0.84	1.19
9	-	0.53	0.43
10	-	0.45	2.00
11	+	0.37	1.07
12	-	0.32	2.00
13	-	0.23	0.30
14	-	0.15	0.43
15	+	0.16	0.76
16	-	0.09	1.74
17	-	0.27	2.00
18	-	0.15	2.00
19	-	0.12	2.00
20	-	0.08	0.05
cut-off		0.27	0.29

Of these 20 samples, 50% are clearly positive with BHC-7 whereas 85% are positive with BHC-9. Two samples (11 & 12) which are borderline positive with BHC-7 are clearly positive with BHC-9 and some of the samples at or below the cut off with BHC-7 are positive with BHC-9. In addition, two samples (11 & 15) which were borderline or negative with BHC-7 but positive with BHC-9 are PCR-positive.
Overall there are only two samples (13 & 20) which are negative with both polypeptides and PCR.

Example 16 -
Isolation of PT-NANBH DNA sequences overlapping existing clones

The immunological screening of cDNA expression libraries described in Examples 3,4 and 14, can only identify those clones which contain an immunoreactive region of the virus. Another approach to the production of clones specific for PT-NANBH is to use PCR to amplify cDNA molecules which overlap the existing clones. Sets of primers can be prepared where one member of the pair lies within existing cloned sequences and the other lies outside; this approach can be extended to nested pairs of primers as well.

cDNA, prepared as described in Example 1, was amplified by PCR, with either single or nested pairs of primers, using the reaction conditions described in Example 13. The approach is illustrated by use of the following pairs of primers; d164 (SEQ ID NO : 12) and d137 (SEQ ID NO : 13); d136 (SEQ ID NO : 14) and d155 (SEQ ID NO : 15); d156 (SEQ ID NO : 16) and d92 (SEQ ID NO : 17). One member of each pair is designed to prime within existing cloned sequences (d137 and d136 prime within the 5′ and 3′ ends of BR11 respectively, d92 primes at the 5′ end of JG3). The other primers are based upon sequences available for other PT-NANBH agents. Primer d164 corresponds to bases 10 to 31 of figure 2 in Okamoto et al, Japan. J. Exp. Med., 1990, 60 167-177. Primers d155 and d156 correspond to positions 462 to 489 and 3315 to 3337 respectively in figure 47 of European Patent Application 88310922.5. One or more nucleotide substitutions were made to

introduce an EcoRl recognition site near the 5' end of the primers, except for d164 where a Bgl2 recognition site was introduced; these changes facilitate the subsequent cloning of the amplified product.

The PCR products were digested with the appropriate restriction enzyme(s), resolved by agarose gel electrophoresis and bands of the expected size were excised and cloned into both plasmid and bacteriophage vectors as described in Example 5. The sequences of the amplified DNAs 164/137 (SEQ ID NO : 18), 136/155 (SEQ ID NO : 19) and 156/92 (SEQ ID NO : 20) are presented in the Sequence Listing. These new sequences extend the coverage of the PT-NANBH genome over that obtained by immunoscreening (SEQ ID NO : 3, 4 & 5). These sequences, together with others which lie within the regions already described, can be combined into a contiguous sequence at the 5' end (SEQ ID NO : 21) and at the 3'-end (SEQ ID NO : 22) of the PT-NANBH genome.

Example 17

Fusion of Different PT-NANBH Antigens into a Single Recombinant Polypeptide

The data presented in Table 7 indicate that whilst more serum samples are detected as antibody-positive using BHC-9 as a target antigen (17/20) rather than BHC-7 (10/20) there are some samples (e.g. #4) which are positive with only BHC-7. This picture is borne out by wider testing of samples. Accordingly, a fusion construct was derived using sequence from BHC-7 and BHC-9.

Sequences from BHC-7 and BHC-9 may be combined in a variety of ways; either sequence may be positioned at the amino terminus of the resulting fusion and the nature of the linking sequence may also be varied. Figure 2 illustrates two possible ways in which the sequences may be combined.

Appropriate restriction fragments carrying suitable restriction enzyme sites and linker sequences were generated either by PCR using specific

primers or by restriction enzyme digestion of existing plasmids. The transfer vector DX143 consists of a BamH1/Pst1 fragment from DX122 (Figure 1; the Pst site is at position 1504 JG2, SEQ ID NO:3) linked to the 5' end of the entire coding region of BR11 (SEQ ID NO:7) which has been amplified as a Pst1/BamH1 fragment using primers d24 (SEQ ID NO:23) and d126 (SEQ ID NO:24); the linkage region consists of six amino acids derived from the d126 primer and residual bacteriophage lambda sequences. The transfer vector DX136 differs from DX143 in that the BR11 fragment was generated using d24 (SEQ ID NO : 23) and d132 (SEQ ID NO : 25) and so the linkage region contains five lysines. These transfer vectors were used to co-transfect Sf9 insect cells in culture with AcNPV DNA and plaque purified stocks of recombinant baculoviruses were produced as described in Example 7. BHC-10 was produced as a result of transfection with DX143; BHC-11 as a result of transfection with DX136.

The recombinant polypeptides expressed by these two viruses were analysed by SDS-PAGE and western blotting. BHC-10 produced a polypeptide with an apparent molecular weight of 118kDa. BHC-11 produced a polypeptide with an apparent molecular weight of 96kDa. Both polypeptides reacted with sera known to react in ELISA only with BHC-7 (e.g. serum A) or only with BHC-9 (serum B64, Example 14). The two polypeptides only differ in the linker sequence and this may affect either their mobility on SDS-PAGE or how they are processed in the infected cells.

Example 18 -
Performance of PT-NANBH Fusion Antigens in an ELISA

An ELISA was established using microtitre wells coated with BHC-9-infected cell extracts and an anti-human Ig conjugate following the procedure described in Example 10. Table 8 presents the data from a comparison of the two fusions with the other PT-NANBH recombinant antigens BHC-7 and BHC-9 as well as the HCV recombinant protein C-100-3 (Ortho Diagnostic Systems, Raritan, New Jersey). The sera are

grouped by pattern of reaction with BHC-7, BHC-9 and C-100-3. Group I sera react strongly with all three antigens; Group II react strongly with only BHC-7; Group III react strongly with only BHC-9 and Group IV react strongly with only two out of the three antigens.

TABLE 8

SERUM	BHC-7	BHC-9	C-100-3	BHC-10	BHC-11
Group I					
AH	>2.0	>2.0	>2.0	>2.0	>2.0
AC	>2.0	>2.0	>2.0	>2.0	>2.0
57	>2.0	>2.0	>2.0	>2.0	>2.0
77	>2.0	>2.0	>2.0	>2.0	>2.0
84	1.4	>2.0	>2.0	>2.0	>2.0
Group II					
805-6	>2.0	0.261	0.1	1.78	+*
805-17	>2.0	0.181	0.12	1.37	+*
805-149	>2.0	0.651	0.084	1.57	++*
Group III					
JS	0.32	>2.0	0.17	>2.0	>2.0
805-57	0.069	1.403	0.25	1.9	+*
805-82	0.116	1.272	0.4	1.85	++*
805-94	0.353	1.675	0.2	>2.0	+*
PJ1	0.27	>2.0	0.2	>2.0	1.85
Group IV					
A	>2.0	0.14	>2.0	>2.0	>2.0
KT	1.57	0.27	>2.0	>2.0	>2.0
Le	0.152	>2.0	>2.0	>2.0	>2.0
PJ5	0.123	>2.0	>2.0	>2.0	>2.0
303-923	>2.0	0.9	0.37	1.9	+*
303-939	>2.0	1.55	0.268	2.0	+*

* These samples have only been tested by western blotting on BHC-11.

These data show that both BHC-10 and BHC-11 have a similar reactivity with these sera and, most importantly, that the both antigenic activities appear to have been retained by the fusions. All the sera in Groups II & III, which react with only BHC-7 or BHC-9 respectively, give a clear reaction with the fusions. Additionally there is an indication that having the two antigens together gives a more sensitive assay. For example the sample KT gives ODs of 1.57 and 0.27 with BHC-7 and BHC-9 respectively whereas with the fusions the OD is >2.0.

MJS/AC/12th December 1990